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Versatile retargeting of SH3 domain binding by modification of non-conserved loop residues

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Abstract **Src-homology (SH3) domain belongs to a class of ubiquitous modular protein domains found in nature. SH3 domains have a conserved surface that recognises proline-rich peptides in ligand proteins, but additional contacts also contribute to binding. Using the SH3 domain of hematopoietic cell kinase as a test case, we show that SH3 binding properties can be profoundly altered by modifications within a hexapeptide sequence in the RT-loop region that is not involved in recognition of currently known consensus SH3 target peptides. These results highlight the role of non-conserved regions in SH3 target selection, and introduce a strategy that may be generally feasible for generating artificial SH3 domains with desired ligand binding properties.**

Keywords: SH3; Phage display; Protein engineering; Signal transduction

1. Introduction

Src-homology (SH3) domains are small protein modules that mediate inter- and intramolecular protein interactions, and are often found in proteins involved in cellular signalling pathways, cytoskeletal organisation, and membrane trafficking (for reviews, see [1,2]). SH3 domains recognise short proline rich sequences typically containing a core PxxP binding motif (where P is proline and x is any amino acid) flanked on either side by a basic residue that determines the orientation of binding. However, several atypical SH3-binding sites lacking a conventional PxxP consensus sequence have also have been described (e.g. [3–5]).

PxxP and other core binding motifs serve as critical docking sites for SH3 domains, but additional molecular contacts outside this conserved interaction interface often help to improve the specificity and affinity of SH3 binding. These additional contacts may involve secondary structures that are adjacent to the PxxP motif in the polypeptide chain of the ligand protein [6,7], but may also be located distantly. A good example of the latter arrangement is provided by the human immunodeficiency virus type-1 (HIV-1) negative factor (Nef) protein. The SH3 domain of hematopoietic cell kinase (Hck) binds strongly to Nef (K_D 0.25 μM), whereas the affinity of the related Src-family kinase SH3 domain of Fyn is much lower [8]. X-ray crystallographic and mutagenesis studies have revealed that residues in the tip of the RT-loop, which is the most variable region among different SH3 domains, account for the strong and specific binding Nef-binding by Hck-SH3 [9,10]. These RT-loop residues of Hck-SH3 are not involved in recognition of the PxxP motif of Nef, and instead contact a hydrophobic pocket on the opposite side, which is formed by multiple residues positioned apart from each other in the Nef polypeptide.

We have shown previously that binding of Hck-SH3 to Nef can be further increased via phage display-mediated optimisation of six residues in this non-conserved RT-loop region [11]. These engineered molecules (dubbed RRT-SH3 for randomised RT-loop) could act as potent inhibitors of Nef function when introduced in cultured cells [12]. In this study we have examined the general feasibility of this strategy, and show that diverse SH3 ligands, including proteins with little or no affinity for unmodified Hck-SH3, can be effectively targeted by such RRT-SH3 domains. These results highlight the contribution of SH3 residues outside of the conserved peptide-binding groove in ligand recognition, and show that manipulation of the RT-loop is a powerful strategy to generate artificial SH3 domains with desired ligand binding properties.

2. Materials and methods

2.1. Expression vectors and recombinant protein purification

To produce the ligands for SH3-phage binding the intracellular domains of CD3ε (residues 131–185) and ADAM15 (residues 711–862, [13]), the amino terminal fragment of p21-activated kinase-1 (PAK1) (residues 1–272), the carboxyterminal fragment of Sos1 (residues 1135–1333), and the complete coding region of the PI3K p85α regulatory subunit were cloned into the pGEX-4T-1 vector (GE Healthcare Bio-Sciences, Uppsala, Sweden). Expression and purification of the resulting GST fusion proteins in *Escherichia coli* were carried out according to manufacturer's instructions. After glutathione elution the proteins were concentrated in PBS with Amicon Ultra 4 columns (Millipore, Billerica, MA, USA).

2.2. Phagemids and phage display

The multivalent library of artificial Hck-derived SH3 domains was created into pG8H6 [14] as described before [15]. Briefly, by using a degenerate PCR primer six amino acid residues of human Hck-SH3

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Abbreviations: SH3, Src-homology; Hck, hematopoietic cell kinase; HIV-1; human immunodeficiency virus type-1; SIV, simian immunodeficiency virus; Nef, negative factor; RRT, randomised RT-loop; PAK1, p21-activated kinase-1

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domain (corresponding to residues 90–95 of human Hck) were randomised and the resulting RRT-SH3 fragments were cloned as fusion proteins to the phage coat protein VIII carrying an N-terminal Myc epitope tag. In addition, a TAG stop codon was placed in front of the RRT-SH3 insert to reduce expression of the fusion protein in a *supE* host (TG1 strain of *E. coli*) in which amber termination is incompletely suppressed. RRT-SH3 clones previously selected with HIV-1 Nef using a monovalent phage system [12] as well as unmodified Hck-SH3 were individually inserted into the multivalent pG8H6 vector by PCR-assisted cloning. Recombinant phages were produced via infection with the M13KO7 helper phage and screened with recombinant fusion proteins immobilised on six-well plates as described previously [11].

2.3. Phage-assisted SH3-binding assay

MaxiSorp wells (Nalge Nunc, Rochester, NY, USA) were coated with 200 ng of purified GST-ligand fusion proteins or plain GST in 50 μl of TBS overnight at 4 °C. Alternatively the wells were coated with 100 ng of anti-Myc antibody 9E10 (Sigma–Aldrich, St. Louis, MO, USA). Wells were washed three times with washing buffer (WB; TBS/0.05% Tween 20) followed by an hour incubation with 2% milk in TBS at room temperature, and washed again three times with WB. Supernatants of the recombinant multivalent phage clones expressing the RRT-SH3 domains fused to a Myc epitope tag (titres $>10^{11}$ pfu/ml) were added to wells and incubated for 2 h at room temperature. The wells were washed three times with WB, and peroxidase conjugated anti-M13 antibody (GE Healthcare Bio-Sciences) was added to wells and incubated for an hour. Wells were washed three times with WB and 50 μl of the ready-to-use substrate 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma–Aldrich) was added per well. The enzymatic reactions were stopped after 30 min by adding 50 μl of 1% SDS, followed by optical density measurement at 405 nm. After background subtraction the readings were normalised based on the corresponding readings from the anti-Myc-coated wells.

3. Results

In our earlier studies aimed at improving binding of Hck-SH3 to HIV-1 and simian immunodeficiency virus (SIV) Nef proteins [11,15] we have generated large mono- and multivalent phage libraries displaying Hck-derived RRT-SH3 domains carrying random hexapeptide sequences in the RT-loop region (Fig. 1). The success in these studies prompted us to investigate the potential of the RT-loop as a general specificity/affinity-determining region in SH3 ligand recognition. To test this idea we selected as potential RRT-SH3 targets a panel of known SH3 ligand proteins that serve important roles in cellular signal transduction, namely CD3ε, ADAM15, PAK1, Sos1, and p85α (the regulatory unit of PI3K), and expressed these as GST fusion proteins in *E. coli*. Previous studies have shown that ADAM15 and PAK1, but not p85α, CD3ε, or Sos1 can bind to Hck-SH3 [13,16]. With the exception of p85α, expression of full-length versions of these pro-

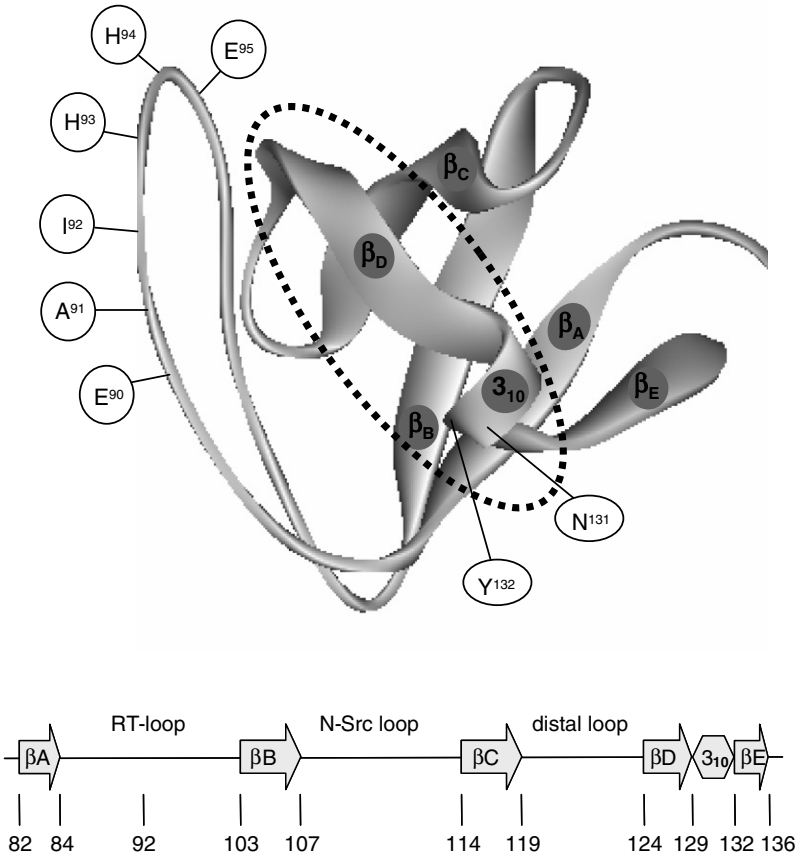

Fig. 1. Structure of the SH3 domain of Hck. The six residues (E^{90}AIHHE^{95}) in the RT-loop subjected to sequence manipulation are indicated. The dashed circle indicates the groove accommodating the core binding peptide of the ligand, and two highly conserved amino acids (N^{131} and Y^{132}) that are key structural components of one of the two proline-binding pockets in this groove are also shown. A schematic representation of the secondary structure of Hck-SH3 is shown below the 3-D structure. Amino acid numbering is according to full-length human Hck. The structure (5HCK; [21]) was obtained from Protein Data Bank and styled with DS Visualizer.

teins in *E. coli* was not feasible (not shown). Therefore, for the remaining ligand proteins the relevant domains harbouring their SH3-binding motifs were expressed. In case of the transmembrane proteins CD3ε and ADAM15 the complete intracellular domain was chosen (residues 131–185 and 711–862, respectively), whereas the regulatory domain of PAK1 (residues 1–272) and the carboxyterminal domain of Sos1 (residues 1135–1333) were used.

Six-well plastic plates were coated with these fusion proteins or plain GST as a control, and incubated with a multivalent phage library expressing approximately 420 million individual RRT-SH3 clones fused to the M13 coat protein pVIII. After washing of the wells, *E. coli* cells were infected by the bound phages, and used to generate a secondary amplified sublibrary via M13KO7 helper virus infection. After four such rounds of selection and amplification, the titre of infectious phages associated with the GST-ligand fusion protein used for their selection was significantly higher than with plain GST tested as a control (data not shown). Several (typically 10) bacterial colonies from each plate were chosen for further analysis. Phagemid DNA was isolated from these colonies to determine the RT-loop sequence in these RRT-SH3 clones. Typically, the RT-loops of two or three of the clones selected using the same ligand were identical, and the remaining clones showed varying degree of similarity to each other (Table 1 and data not shown).

Individual infectious phage supernatants were generated from each unique clone, and their ability to bind to their cognate ligands was confirmed based on counting of target protein-associated infectious phage titres as above. In all cases, these clonal phage preparations bound to the protein used for their selection much better than to plain GST (not shown). Table 1 shows the RT-loop hexapeptide found in place of the natural Hck-SH3 sequence (EAIHHE) in five RRT-SH3 domains, which based on this initial functional characterisation appeared to bind particularly well to their target protein. For comparison Table 1 also shows RT-loop sequences of five RRT-SH3 clones previously derived via extensive selection of optimal binders to HIV-1 Nef from a monovalent phage library [11]. To include these Nef-targeted SH3 domains for comparison into studies aimed at functional characterisation of the novel RRT-SH3 clones, the former were also cloned into the same multivalent M13 phage system used for affinity selection in this study.

Similar to our earlier work on HIV-1 and SIV Nef proteins [11,15], RT-loop sequences of RRT-SH3 domains selected with a given ligand protein were heterogeneous, but yet showed significant homology to each other (see Table 1). By contrast, the sets of RRT-SH3 domains selected with different ligands showed little similarity to each other, or to those selected earlier with HIV-1 Nef. The only exception in this regard was that a glycine residue often appeared in the last randomised RT-loop position in RRT-SH3 domains targeted for binding to ADAM15, PAK1, Sosl, and p85α. None of these hexapeptides matched with an RT-loop sequence found in a natural SH3 domain.

Three RRT-SH3 domains targeted against each ligand protein were selected for more detailed functional studies. To characterise their binding properties we developed a semi-quantitative interaction assay similar to that described by Winter and colleagues [17]. As illustrated in Fig. 2, this assay was based on recognition of immobilised SH3 ligand proteins by

Table 1
RT-loop sequences found in RRT-SH3 domains selected for optimal binding to different ligands

Ligand	SH3 domain	RT-loop
–	Hck SH3	EAIHHE
Nef	A1	VSWSPD
	A2	FSWSDT
	B4	FSPFDW
	B6	YSPFSW
	C1	TSPFPW
PAK1	P1	GGLYAD
	P2	MPLYVG
	P3	NPLYVG
	P4	LPLYMG
	P5	DAVYMD
ADAM15	15.1	TGEDRN
	15.2	IAYGDG
	15.3	MAQCIG
	15.4	RAEQSG
	15.5	DGVLVG
CD3ε	ε1	WGRNAM
	ε2	LATNRY
	ε3	TRANVF
	ε4	WGRNRL
	ε5	VARATP
Sos1	S1	GAMEVG
	S2	VGSEYD
	S3	REVYEG
	S4	QEEMPG
	S5	RALELG
PI3K p85α	85.1	VGTEYG
	85.2	WAEEVG
	85.3	EALEYG
	85.4	MALWEG
	85.5	GGLYWD

Shown is the hexapeptide region subjected to modifications in Hck-SH3 (see Fig. 1), and the corresponding residues in RRT-SH3 clones targeted for binding to the indicated ligands.

RRT-SH3 domains expressed on the surface of phage particles, followed by detection of the bound SH3 domains using an enzyme-conjugated anti-phage antibody. Following determination and normalisation of the infectious titre of these phages, the amount of RRT-SH3 domains available for ligand-specific binding on their surface was determined by measuring in parallel the efficiency of capture of the same phages by an immobilised monoclonal antibody against a Myc peptide epitope present in each phage between the RRT-SH3 domain and the pVIII coat protein (see Fig. 2). The ratio of specific ligand binding vs. anti-Myc binding was then used as a measure of the relative affinity of the displayed RRT-SH3 domains for their cognate ligands.

As shown in Fig. 3, in the case of each of the five ligand proteins (CD3ε, ADAM15, PAK1, Sos1, and p85α) all three selected RRT-SH3 domains showed substantially improved binding when compared to native Hck-SH3. This improvement was most striking in the case of CD3ε and p85α, which bound avidly to their cognate RRT-SH3 domains, but only minimally or not at all to Hck-SH3. Similar to the case with Nef, however, RT-loop manipulation could also provide a significant increase in affinity towards ADAM15 and PAK1, which could bind relatively well also to native Hck-SH3.

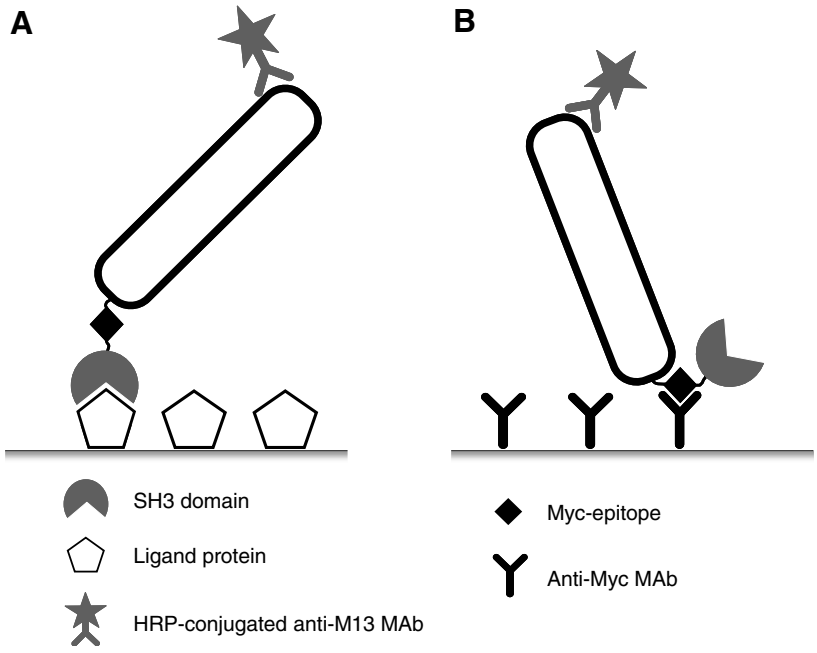

Fig. 2. Principle of the phage-assisted SH3-binding assay. SH3 ligand proteins of interest (A) or anti-Myc antibodies (B) were immobilised to plastic wells that were subsequently incubated with different dilutions of homogenous phage preparations expressing on their surface SH3 domains fused to a Myc epitope peptide. In both cases (A and B) binding of the SH3-displaying phage particles to these wells was measured based on a chromogenic reaction catalysed by a peroxidase-conjugated anti-phage antibody. The ratio of binding signals obtained in assay configuration A vs. B was considered as the measure of specific SH3-binding capacity of the displayed SH3 clone.

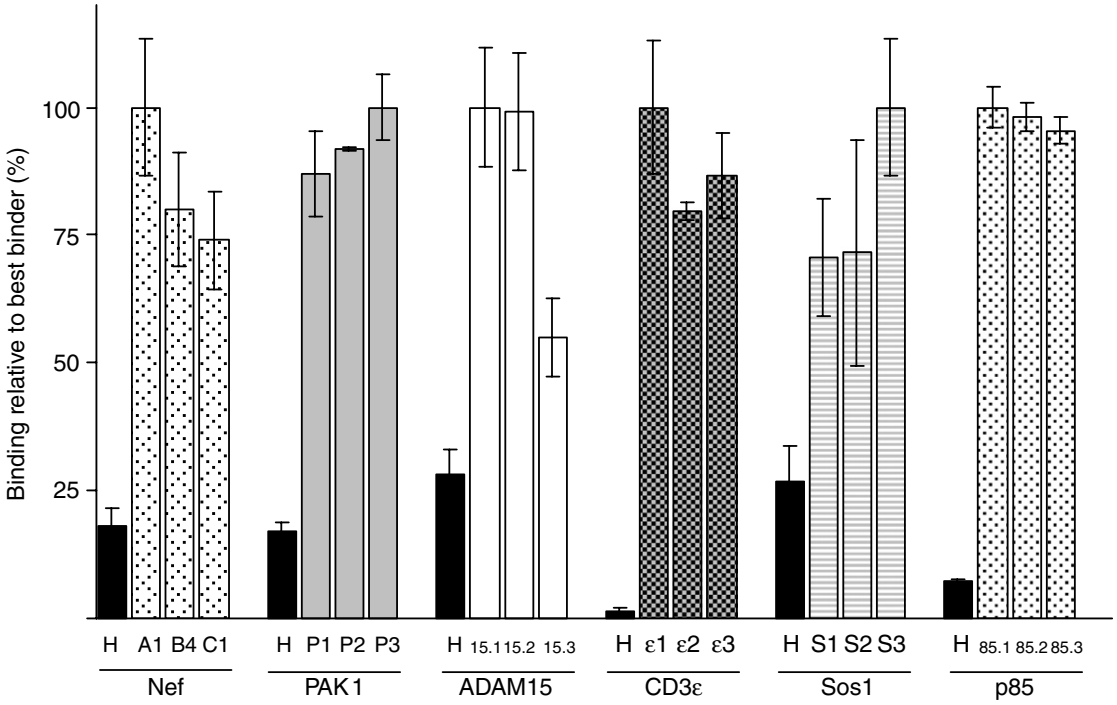

Fig. 3. Relative increase in binding of RRT-SH3 domains to their cognate ligands compared to unmodified Hck-SH3. Three RRT-SH3 domains selected for binding to PAK1 (P1 – P3), ADAM15 (15.1–15.3), CD3ε (ε1–ε3), Sos1 (S1–S3), or p85α (85.1–85.3) were compared with Hck-SH3 in binding to their target proteins using the assay shown in Fig. 2. For comparison, multivalent phage particles carrying three RRT-SH3 domains (A1, B4, and C1) previously selected for optimal binding to Nef from a monovalent phage library [11] were generated and tested in parallel for their binding to Nef. In the case of each SH3 target protein the strongest binding signal observed was set to 100%, and binding of Hck-SH3 and the other two RRT-SH3 domains was expressed relative to this. An average of at least three independent experiments is shown. Interassay variation is indicated by the error bars.

Due to the nature of this assay it was not possible to determine the absolute binding affinities of these interactions. For comparison, however, it is worth noting that when a more quantitative assay based on purified recombinant proteins was used to examine binding properties of the RRT-SH3 domains A1, B4, and C1, they showed a 21- to 35-fold increased capacity in binding to HIV-1 Nef compared to native Hck-SH3 [11]. Considering that this was reflected only by a 3- to 5-fold increase in the binding signal of the current assay, the relative increase in the capacity of these RRT-SH3 domains to bind to CD3ε, ADAM15, PAK1, Sos1, and p85α could very well be more pronounced than apparent from the data shown in Fig. 3.

Although our results revealed room for further biotechnological optimisation, the natural RT-loop of Hck-SH3 plays an important role in mediating the molecular contacts that account for the strong binding to Nef (K_D 250 nM; [8]). Therefore, one could predict that artificial targeting of Hck-SH3 for increased binding to CD3ε, ADAM15, PAK1, Sos1, or p85α via RT-manipulation would lead to a reciprocal loss in affinity for Nef. To test this hypothesis we used the phage-based assay shown in Fig. 2 to examine the capacity these novel RRT-SH3 domains to bind to Nef. As shown in Fig. 4, all RRT-SH3 clones selected by non-Nef ligand proteins were indeed poorly competent for binding to Nef. Only two ADAM15-selected clones (15.2 and 15.3) and one PAK1-selected clone (P3) showed binding to Nef that was above 10% of the signal observed for native Hck-SH3, and in many cases no detectable Nef-binding could be observed. Thus, the amino acid changes in these RRT-SH3 domains that provided them with increased affinity towards their targeted ligands led to diminished affinity towards Nef and presumably towards other natural interactions of Hck-SH3 where the RT-loop is involved.

Various proteins scaffolds have been used to engineer and display short peptides that can independently bind to ligands of interest (see [18]), and recently the SH3-fold was also shown to be useful for this purpose [19]. We have previously shown that despite their highly increased affinity for Nef, the RT-loop-modified SH3 domains A1, B4, and C1 are still fully dependent on canonical docking of the Hck-SH3 core region to the consensus PxxP motif-containing binding site in Nef, and cannot interact with a Nef mutant carrying a PxxP-to-AxxA substitution [11]. Nevertheless, we wanted to make sure that the novel RRT-SH3 domains generated in this study behaved as *bona fide* SH3 domains, and the altered RT-loop sequences that provided them with novel binding properties did not act independently of the rest of these SH3 domains. Since many of the ligand proteins used in this study contained multiple potential PxxP target sites, we used an alternative approach to prevent docking of the RRT-SH3 domains to proline-rich target peptides by introducing in these SH3 clones mutations that disrupted one of the two proline binding pockets of Hck-SH3. To this end we replaced both Asn^{131} and Tyr^{132} of Hck with alanine residues, or alternatively mutated Tyr^{132} alone by introducing a more radical change, namely a negatively changed residue (aspartic acid) that is not found in this position in any human SH3 domain (see Fig. 1). Such mutants were generated of two individual RRT-SH3 clones targeted for binding to CD3ε (ε1 and ε3), ADAM15 (15.1 and 15.2), PAK1 (P1 and P2), p85 (85.1 and 85.2) and Sos1 (S1 and S2), and their capacity to bind to their cognate ligand was tested using the same assays as above. None of the recombinant phages expressing either NY-to-AA or NY-to-ND mutated RRT-SH3 domains showed any binding to their cognate ligand proteins (data not shown). Thus, we conclude that the radically altered binding properties of the modified Hck-SH3 derivative developed in this study can be attributed to a prominent, but

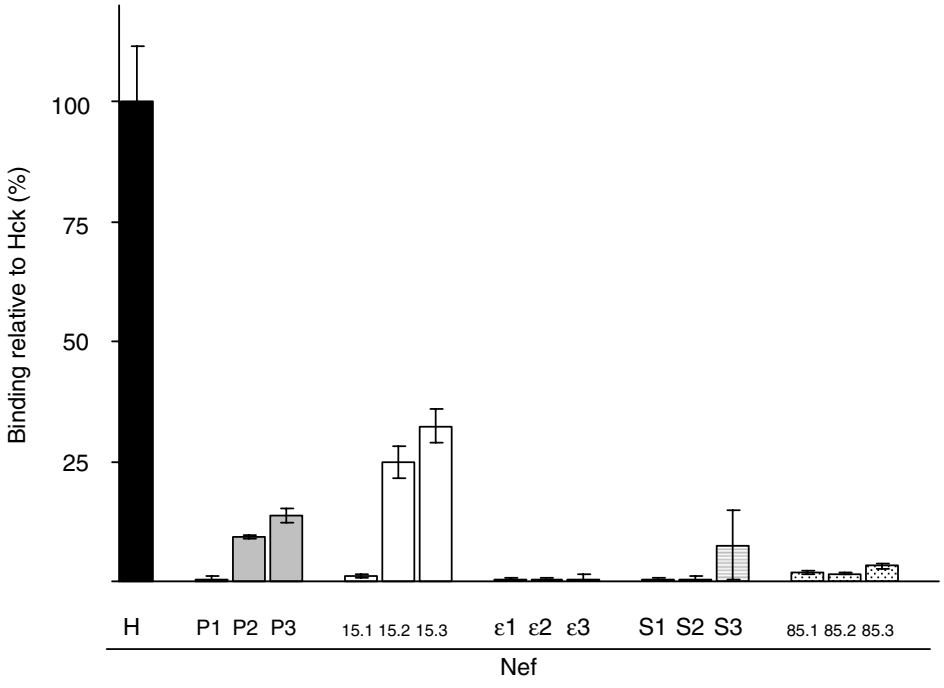

Fig. 4. Loss of Nef-binding capacity relative to native Hck-SH3 of RRT-SH3 domains optimised for binding to PAK1, ADAM15, CD3ε, Sos1, or p85α. The RRT-SH3 domains characterised in Fig. 3 for specific binding were tested in parallel with unmodified Hck-SH3 for binding to HIV-1 Nef, a natural high-affinity SH3-binding partner of Hck. Measurements and data management was done as in Fig. 3.

yet not SH3 independent, capacity of the RT-loop to modify ligand selection.

4. Discussion

In this study we have shown that manipulation of six residues in the non-conserved tip region of the RT-loop of Hck-SH3 is sufficient to dramatically alter its ligand binding specificity. The increased affinity of Hck-SH3 towards the targeted ligands was particularly striking in case of CD3ε and p85α, which showed no detectable binding to unmodified Hck-SH3. This improved binding was in all cases accompanied by a reciprocal loss in the ability of these engineered SH3 domains to bind to HIV-1 Nef, a natural high-affinity ligand of Hck-SH3.

The experimental system used did not allow us to determine the absolute binding affinities of the engineered RRT-SH3 domains towards their ligands. Based on a rough comparison with the apparent strength of Nef-binding by native Hck-SH3 and the Nef-targeted RRT-SH3 domains A1, B4, and C1 it is likely that the affinities of these interactions are in a low nanomolar range or below, which indicates binding that is tighter than seen in most natural SH3/ligand complexes. However, knowing the precise affinity values is not critical for the present conclusions, which are fully supported by the dramatic and reciprocal relative changes observed in the strength of binding of Hck-SH3 to the targeted (CD3ε, ADAM15, PAK1, Sos1, or p85α) ligands vs. a natural high affinity partner (HIV-1 Nef) that could be achieved entirely via RT-loop manipulation.

The failure of a given SH3 domain to interact with a protein that binds avidly to another SH3 has generally been thought to result from a poor fit between the proline-rich region of the ligand and the peptide-binding groove of the former SH3. Although the proximal regions of the RT-loop participate in formation of the conserved SH3 peptide-binding surface, it should be emphasised that the engineered residues unique to each RRT-SH3 domain are located in the central tip region of the RT-loop apart from the peptide-binding groove of Hck-SH3 (see Fig. 1). Thus, our results extend this traditional view of SH3 binding, and reveal a striking capacity of ''additional'' or ''tertiary'' interactions to influence SH3 ligand selection.

The implications of such a potentially dominating role of the RT-loop (and perhaps other divergent loops in the SH3-fold) over the consensus SH3 peptide docking surface are 2-fold. First, the relevance of any conclusions regarding preferred SH3-mediated protein interactions based on data on SH3 binding by isolated consensus peptide ligands might be limited. In other words, because the strength and specificity of binding may predominantly depend on molecular determinants outside of the conserved SH3/peptide interface, differences in the affinity of this core interaction can in such cases not be used to accurately predict the relative SH3-binding affinities of the corresponding native ligand proteins.

Second, our results show that RT-loop manipulation may be a generally feasible strategy for engineering of SH3 domains with rationally altered ligand binding properties. We have previously shown that RRT-SH3 domains optimised for binding to HIV-1 Nef can be used as potent intracellular inhibitors of Nef function [12]. If necessary, the inhibitory potential of RRT-SH3 domains might be further increased by fusing these with other functional domains that could actively induce mislocalisation or degradation of the complexed ligand. Delivery of such inhibitory RRT-SH3 constructs using gene transfer vectors could complement therapeutic strategies currently being developed for inhibition of cellular signalling proteins based on small molecular molecules or RNAi-mediated approaches.

Perhaps even more useful than the immediate inhibitory potential of RRT-SH3 domains might be the possibility to use their altered ligand specificities for reprogramming of cellular signal transduction cascades. In elegant experiments by Lim and colleagues protein engineering was used to create artificial signalling proteins composed of a selected set of modular domains derived from heterologous proteins [20]. These rationally designed proteins could carry out sophisticated behaviours that are essential for signalling proteins operating in complex cellular circuits, such as allosteric gating and multi-input signal integration, but in a non-physiological manner that logically reflected their artificial compositions. SH3 domain belongs to a class of ubiquitous modular protein domains found in nature, and therefore is a useful building block for such synthetic switch proteins (a term coined by the Lim laboratory) or related artificial signalling proteins. The ability to manipulate the affinity and selectivity of SH3 ligand recognition via RT-loop engineering provides a powerful tool for enhancing such efforts.

The RT-loop sequences of the RRT-SH3 domains targeted for binding to CD3ε, ADAM15, PAK1, Sos1, or p85α did not closely resemble any naturally occurring SH3 domain. Nevertheless, the molecular contacts used by the engineered RRT-SH3 domains could be optimised but yet essentially similar to those used by the corresponding RT-loop residues of natural SH3 domains upon binding to the same ligands. Conversely, it is also possible that (at least in some cases) the RT-loops of these RRT-SH3 domains interacted with their ligands in a less natural manner, meaning that the target determinants in the ligand would normally not be involved in SH3 binding. Such speculations are of interest when thinking of the possible role of the RT-loop as a general specificity and affinity determinant in SH3-mediated interactions in nature, but are less relevant for the concept of exploiting ligand-targeted RRT-SH3 domains as inhibitors or modifiers of intracellular signalling pathways.

The surprising flexibility and potential dominance of the RT-loop in determining SH3 ligand selection demonstrated here using the SH3 domain of Hck as a test case suggest that this approach could be used to target with high affinity the majority of cellular and microbial SH3 ligands. However, to cover the full repertoire of SH3 target proteins, including those containing highly atypical binding motifs, a panel of randomised libraries based on a set of divergent members of the SH3 domain family should perhaps be constructed. Moreover, with the increasing knowledge about structure-function aspects of ligand recognition by modular protein binding domains other than SH3, the same approach might be extended to target many more protein interactions, which could further improve the tool box available for rational design of modulators of cellular signalling to be used in experimental and possibly therapeutic applications.

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